Parental age affects somatic mutation rates in the progeny of flowering plants

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Abstract: In humans, it is well known that the parental reproductive age has a strong influence on mutations transmitted to their progeny. Meiotic nondisjunction is known to increase in older mothers, and base substitutions tend to go up with paternal reproductive age. Hence, it is clear that the germinal mutation rates are a function of both maternal and paternal ages in humans. In contrast, it is unknown whether the parental reproductive age has an effect on somatic mutation rates in the progeny, because these are rare and difficult to detect. To address this question, we took advantage of the plant model system Arabidopsis (Arabidopsis thaliana), where mutation detector lines allow for an easy quantitation of somatic mutations, to test the effect of parental age on somatic mutation rates in the progeny. Although we found no significant effect of parental age on base substitutions, we found that frameshift mutations and transposition events increased in the progeny of older parents, an effect that is stronger through the maternal line. In contrast, intrachromosomal recombination events in the progeny decrease with the age of the parents in a parent-of-origin-dependent manner. Our results clearly show that parental reproductive age affects somatic mutation rates in the progeny and, thus, that some form of age-dependent information, which affects the frequency of double-strand breaks and possibly other processes involved in maintaining genome integrity, is transmitted through the gametes.

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Parental Age Affects Somatic Mutation Rates in the Progeny of Flowering Plants

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Using a set of mutation detector lines, we found that in Arabidopsis the reproductive age of the parents has a significant influence on the kind and rate of somatic mutations in their progeny.

ABSTRACT

In humans, it is well known that the parental reproductive age has a strong influence on mutations transmitted to their progeny. Meiotic non-disjunction is known to increase in older mothers and base substitutions tend to go up with paternal reproductive age. Hence, it is clear that the germinal mutation rates are a function of both maternal and paternal age in humans. In contrast, it is unknown whether the parental reproductive age has an effect on somatic mutation rates in the progeny, as these are rare and difficult to detect. To address this question, we took advantage of the plant model system *Arabidopsis thaliana*, where mutation detector lines allow for an easy quantitation of somatic mutations, to test the effect of parental age on somatic mutation rates in the progeny. While we found no significant effect of parental age on base substitutions, frameshift mutations and transposition events increased in the progeny of older parents, an effect that is stronger through the maternal line. In contrast, intra-chromosomal recombination (ICR) events in the progeny decrease with the age of the parents in a parent-of-origin-dependent manner. Our results clearly show that parental reproductive age affects somatic mutation rates in the progeny and, thus, that some form of age-dependent information is transmitted through the gametes, which affects the frequency of double strand breaks (DSB).

INTRODUCTION

In humans, it has long been recognised that the reproductive age of the parents has an influence on the health of their progeny. An older reproductive age of the mother is known to increase the fraction of aneuploid gamete formation (Hurles, 2012). For instance, the risk for a trisomy increases from 2-3% for mothers in their twenties to more than 30% for mothers in their forties (Hassold & Hunt, 2009). The age of the father has also an effect on the frequency of spontaneous congenital disorders and common complex diseases, such as autism and some cancers (Goriely and Wilkie, 2012). Indeed, sperm from 36-57 year old men have more double strand breaks (DSBs) than those of 20-35 year old individuals (Singh et al., 2003). Similarly, the efficiency of DSB repair was reported to decrease with age in vegetative tissues of Arabidopsis (Boyko et al., 2006).

Owing to the continuous divisions of spermatogonial stem cells, the male germline of humans is thought to be more mutagenic than the female germline. Indeed, it was shown that the paternal germline is more mutagenic than the maternal one with respect to base substitutions (Kong et al., 2012) and replication slippage errors at microsatellites (Sun et al., 2012). It is also known that carriers of germline mutations in mismatch repair (MMR) genes in humans are more prone to get colorectal cancer and the risk depends on the parent-of-origin of the mutation (Van Vliet et al., 2011). The molecular basis of these

parental effects is not entirely clear but is likely to involve higher rates of non-disjunction during female meiosis, higher mutation rates during spermatogenesis, and probably additional effects of aging.

In contrast to the effect of parental age on germline mutations, not much is known about potential effects of parental reproductive age on somatic mutation rates in the offspring. However, it has been shown in animal studies that radiation of males can lead to somatic mutations in their progeny – and in subsequent generations – that cannot be attributed to mutations in the paternal germline (reviewed in Little et al., 2013). Moreover, several recent studies have illustrated the existence of complex parental and transgenerational effects in humans, although their molecular basis is not clear (Grossniklaus et al., 2013). These effects can either be of genetic nature, but the effect is seen even in offspring that did not inherit the genetic variant from their parents (reviewed in Nadeau, 2009), or of epigenetic nature, where environmental influences can possibly exert effects on subsequent generations (reviewed in Prembrey et al., 2006; Prembrey, 2010; Curley et al., 2011). It is currently not known whether such parental effects affect the somatic mutation rates in the offspring or whether the effects are modulated by parental age.

Taking advantage of the plant model system *Arabidopsis thaliana*, in which various somatic mutation rates can readily be assessed (Bashir et al., 2014), we investigated the effects of parental reproductive age on somatic mutation rates in the progeny. We report that there is a pronounced effect of parental age on somatic mutation rates in their offspring in a parent-of-origin-dependent fashion. Thus, some form of parental information, which is inherited via the gametes to the next generation, seems to alter the somatic mutation rates in the progeny and changes with parental reproductive age.

RESULTS

To study the effect of parental reproductive age on somatic mutation rates in the progeny, we made use of various Arabidopsis transgenic lines carrying mutated or fragmented versions of the *uidA* reporter gene encoding β-glucuronidase (GUS) (Liu and Crawford, 1998; Kovalchuk et al., 2000; Li et al., 2004; Azaiez et al., 2006; Van der Auwera et al., 2008). These mutation reporter lines carrying a non-functional *uidA* gene enabled us to score somatic frameshift mutations, base substitutions, intra-chromosomal recombination (ICR) and transpositions events, which led to the formation of a functional *uidA* gene. GUS activity, reflecting a mutation event, can easily be monitored *in planta* using the chromogenic substrate X-Gluc, (Jefferson *et al.* 1987), the product of which can be detected as blue spots (Fig. S1). By scoring the number of these events in large plant populations, we calculated the rates of these various kinds of mutations (blue spots per plant). The age of the plant was counted from the day the

seeds were plated on MS medium plates for germination. Independent plants were manually self- or cross-pollinated on four separate occasions representing different age groups.

Mutation Rate Estimation after Normalizing for Variation in Cell Number and Ploidy

Reciprocal C24 x Columbia (Col) Arabidopsis hybrids were reported to have differences in the average cell size and cell number (Fujimoto et al., 2012). Also, hybridization has been shown to alter the cell number and average ploidy per nucleus (Bashir et al, 2014). Therefore, it is important to normalize mutation rates by factoring in cell number, cell size, and average ploidy per nucleus in the progenies derived from parents of different age. For mutation rate estimations, we considered four different parental ages, i.e. 38, 43, 48 and 53 days after sowing (DAS) on MS medium. We used the 4^{th} true leaf (excluding the cotyledons) of Col wild-type plants to measure cell size and cell number. As leaf size is largely determined by the epidermis (Savaldi-Goldstein et al., 2007; Marcotrigiano, 2010), the number of cells in the adaxial epidermal surface of the leaves were counted in a specified area using scanning electron micrographs. We found no significant variation in cell size among the progenies derived from parents of different age (Fig. 1A; 1B; 1E). In contrast, the adaxial epidermal cell number decreased with parental age (Fig. 1F). The progeny of 53-day old parents had a considerably lower cell number compared to that of 38-day old self-fertilized parents ($P < 0.001$) (Fig. 1F). Reciprocal crosses 38x48, 48x38 and 43x53, 53x43 revealed a strong effect on leaf surface area, with progenies from older females having a lower cell number compared to those of younger females ($P < 0.001$) (Fig. 1F). With the increase in parental and female reproductive age, we found the average leaf surface area to get smaller (Fig 1C; 1D: 1F). The 4^{th} leaf from 48x38 and 53x43 had a smaller surface area compared to 38x48 and 43x53, respectively ($P < 0.001$), indicating that older females have a strong influence on the total leaf surface area of the progeny (Supplemental Fig. S2).

The calculation of mutation rates is based on the reversion of a single *uidA* gene in the genome. Endoreduplication occurs in 90% of angiosperm species (D' Amato, 1994), and it is known that the level of endoreduplication increases with age, such that the ploidy level is highest in the oldest plants (Melaragno et al., 1993). As this could have an influence on their progeny, it is essential to normalize to the number of genomes per nucleus in the progenies derived from parents of different age. We observed no significant variation in the percentage of nuclei with 2X, 4X and 8X ploidy levels among the progenies from crosses of parents at different age (Fig. 2A), and there was no significant difference in the average ploidy per nucleus in the progenies of parents at different age (Fig. 2B). Similarly, there was no significant difference in the average ploidy in progenies derived from older mothers (48x38 and 53x43) compared to that of younger ones (38x48 and 43x53) (Fig 2B).

Taken together these data show a significant decrease in cell number in the progenies both with increasing parental age and with female reproductive age. Accordingly, mutation rates were corrected for variation in genome size (Table 1) by taking into account the average ploidy per nucleus and the cell number in the progenies derived from different parental ages.

Table 1. *Normalization of mutation rates by factoring differences in cell number and ploidy per nucleus.*

Progenies of different parents were analyzed for their adaxial epidermal cell count and their ploidy per leaf cell nucleus to obtain the correction factor. The coefficient of interquartile range (CIQR) was calculated as a nonparametric measurement of variance in the style of the coefficient of variance (CV). The relative number of cells and the relative ploidy are the normalization values for older parental age (43, 48 and 53 days after sowing (DAS)) compared with youngest parental age (38 DAS) and for reciprocal crosses, the female with higher age (48 x 38 and 53 x 43) was compared with younger age (38 x 48 and 43 x 53). The correction factor was calculated by multiplying the two normalization values. This correction factor was used to correct the number of GUS spots before analysis. n – Number of plants analyzed; Median – median of measurements; CIQR – interquartile range/median.

	Epidermal Cells				Ploidy Per Nucleus				
Age	n	Median	CIQR	Relative number of cells	n	Median	CIQR	Relative ploidy	Correction Factor
38 x 38	4	10645.798	0.028	1	4	3.695	0.081	1	1
43 x 43	8	9086.439	0.091	0.854	7	3.824	0.085	1.035	0.884
48 x 48	6	9229.38	0.282	0.867	7	3.666	0.056	0.992	0.86
53 x 53	8	7568.795	0.182	0.711	5	3.548	0.043	0.96	0.683
38 x 48	8	11781.776	0.142	1	7	3.805	0.076	1	1
48 x 38	8	8039.422	0.313	0.682	6	3.596	0.078	0.945	0.644
43 x 53	15	10463.692	0.15	1	7	3.621	0.17	1	1
53 x 43	16	6485.826	0.204	0.62	6	3.537	0.11	0.977	0.606

Parental Age Increases Frameshift Mutation Rates in the Progeny

In order to assess the effect of parental reproductive age on frameshift mutation rates in the progeny, Col plants containing an out-of-frame mononucleotide guanine repeat (G10) in the *uidA* reporter gene (Azaiez et al., 2006) were used for crosses between parents of different age. Frameshift mutations occurring in this repeat can restore the function of the *uidA* gene due to the addition or deletion of guanine bases. The crosses were carried out soon after the plants started flowering but the very first set of flowers

153 formed were not used as they are often (partially) sterile. For the first pollinations, we used flowers from
154 plants 35-38 days after sowing (DAS) and crosses were performed over a period of about two weeks.
155 As the parental age increased from 38 DAS to 53 DAS, we observed a rise in the frequency of
156 frameshift mutation events in the progeny ($P < 0.001$, Fig. 3). We would like to point out that the progeny
157 from plants both 49 and 53 DAS displayed a significant increase in frameshift mutation events in
158 comparison to the progeny of younger parents (38 and 43 DAS). Interestingly, in reciprocal crosses with
159 plants of different age (38 and 49 DAS), we observed a significant increase ($P < 0.001$) of frameshift
160 mutation events with the age of the mother (Fig. 3). Similar results were obtained when the age of the
161 parents in reciprocal crosses was 53 and 43 DAS ($P < 0.001$).
162 These results clearly indicate that the framshift mutation rate in the progeny goes up with the
163 reproductive age of the parents and that the age of the female parent contributes more towards this
164 increase.
165
166 **Parental Reproductive Age Has no Effect on Base Substitution Rates in the Progeny**
167 To study the effect of parental reproductive age on base substitution rates in the progeny, we used
168 two transgenic lines that allowed us to score for T to G transversion and C to T transition mutations,
169 respectively (Kovalchuk et al., 2000; Van der Auwera et al., 2008). In line $166_{G\rightarrow T}$ G is mutated to T at
170 the 166^{th} position and in line $1390_{T\rightarrow C}$ T is mutated to C at the 1390^{th} position in the ORF of the *uidA*
171 gene, respectively.
172 As the age of the parents increased from 38 to 53 DAS, there was no significant difference in T to
173 G transversion (Fig. 4A) nor in C to T transition rates (Fig. 4B). Neither did we observe significant
174 changes in base substitution rates in reciprocal crosses with parents aged 38 and 49 DAS and 43 and 53
175 DAS, respectively (Fig. 4). Only a trend of somewhat higher C to T transition rates was observed as
176 parental age increased from 38 to 53 DAS (38 DAS vs 49 DAS, $P=0.08$; 38 DAS vs 53 DAS, $P=0.06$; 43
177 DAS vs 53 DAS, $P=0.07$) (Fig. 4B). A trend for a difference in T to G transversions in reciprocal crosses
178 with slightly higher mutation rates in progenies of older mothers was also observed (43x53 vs 53x43,
179 $P=0.06$) (Fig. 4A).
180 In summary, we did not observe any significant changes in filial base substitution rates with
181 increased parental reproductive age, although trends may indicate a slight increase with parental,
182 particularly maternal, age.
183
184 **Parental Reproductive Age Influences the Rate of Intra-Chromosomal Recombination Events in**
185 **the Progeny**

To estimate the effect of ageing on somatic intra-chromosomal recombination (ICR) frequencies in the progeny we used transgenic lines R2L1 and R3L30, carrying two inverted catalase introns within the *uidA* gene. A recombination event within the identical sequences of the catalase introns generates a functional *uidA* gene, resulting in GUS activity that can be visualized as blue spots upon histochemical staining (Li *et al*., 2004).

Using line R2L1, which has a 418 bp inverted intron, ICR rates in the progeny were neither significantly affected by parental reproductive age nor did they significantly differ in reciprocal crosses (Fig. 5A). However, using line R3L30, which has a longer inverted intron of 589 bp, the progeny of 53 DAS old plants displayed a significant drop in ICR events in comparison to that in the progeny of plants 38 DAS ($P < 0.001$), 43 DAS ($P < 0.01$), and 48 DAS ($P < 0.01$) (Fig. 5B). Interestingly, reciprocal crosses also showed a difference in ICR rates depending on the parental age of the parents. For instance, progeny of 53 and 48 DAS old females crossed with 43 and 38 DAS old males, respectively, showed a significant increase in ICR rates compared to the reciprocal crosses ($P < 0.001$ and $P < 0.05$, respectively) (Fig. 5B). The discrepancy between the results obtained with the two lines may be due to the different size of the homologous region or, more likely, due to the genomic neighborhood of the insertion site, which may be reduce the effects of age in line R2L1.

In summary, parental age seems to have a strong effect on ICR rates in the progeny, which may depend on the size of the inverted intron and/or position effects. Similar to what we observed for frameshift mutation rates, the age of the female parent has a stronger influence on the rate of ICR events in the progeny than that of the male parent.

Transposition Rates Increase in Seedlings Derived from Older Parents

To score transposition rates, we used plants carrying the endogenous transposable element *Tag1* inserted between the CaMV 35S promoter and the *uidA* gene, which renders it inactive (Liu et al., 1998). Excision of the *Tag1* element allows expression of the *uidA* gene under the control of the 35S promoter, leading to blue spots in histochemical assays.

We observed a gradual increase of transposition rates in the progeny as parental reproductive age increased, e.g. the transposition rates increased in the offspring of 49 DAS ($P < 0.05$) and 54 DAS old plants ($P < 0.001$) by 71% and 349%, respectively, in comparison to the progeny of younger parents (38 DAS, Fig. 6). Offspring derived from reciprocal crosses involving parents of different age also showed a significant difference in transposition frequencies ($P < 0.001$).

In summary, these results show that the rate of *Tag1* transposition in the progeny increases with parental reproductive age, with a stronger effect through the maternal line.

Seedlings of Older Parents Show a Significant Increase in Double Strand Breaks

To gain some insights into the molecular basis of altered somatic mutation frequencies in seedlings from parents of different age, we analyzed whether the frequency of DSBs was affected. To this aim, we performed a neutral comet assay, which is a single cell gel electrophoresis technique to quantify DNA damage. During electrophoresis, damaged DNA migrates differently from intact DNA and forms the tail of comet-like structure. The extent of DNA damage can be estimated by the length of the comet tail and its fluorescence intensity relative to that of the comet's head (henceforth referred to percentage of tail DNA).

The neutral comet assay allows the detection of double DSBs independent of the presence of single strand breaks (Olive et al., 1991). Intact nuclei from seedlings derived from parents of different age were isolated, and the comet assay was performed using a commercially available kit. With an increase in parental age, we also found a significant increase in the percentage of tail DNA ($P < 0.05$), and this increase depended more on female than the male reproductive age based on reciprocal crosses ($P < 0.001$), (Fig. 7).

In summary, these results indicate that the frequency of DSBs increases in the progeny of older plants, with a more pronounced effect of the mother's age.

DISCUSSION

Parental age is a major determinant for chromosomal aberrations and other mutations in many organisms. How parental age affects mutation rates in their offspring, however, has rarely been investigated. In plants, there is a chance that somatic mutations are transmitted to the next generation and, if the frequencies of such spontaneous events are high from plants derived from older parents, this could give rise to increased variation in future generations. Thus, differences in reproductive age between populations could affect their adaptive potential.

As the level of endoreduplication increases in many organs of both plants and animals with the age of the organism, it is important to take into account the ploidy level, cell size, and cell number (Table 1) for a precise estimation of the mutation rates across progenies from parents of different age groups. We found that leaves become significantly smaller with increasing maternal age. Similarly, in mice three months old male progeny of middle-aged mothers are considerably smaller than those of young mothers (Wang and vom Saal, 2000). In fact, effects of maternal age on offspring size is wide-spread in plants and

animals (Kindsvater and Otto, 2014, and references therein) but the underlying mechanisms vary and are often not well understood.

Our study shows that parental reproductive age affects the somatic mutation rates in a parent-of-origin-dependent fashion in the progeny. One possible explanation is that certain unknown bioactive compounds or epigenetic changes that accumulate with age are inherited to the progeny and affect somatic mutation rates. Previous work had revealed that DNA polymerase activity decreases in older plants (Bottomley, 1970; Golubov et al., 2010) and, if this age-related down-regulation is epigenetically transmitted to the progeny, it may affect somatic mutation rates. Such a down-regulation of gene expression may result from the inheritance of DNA methylation patterns, histone modifications, or small RNAs that mediate silencing (Brennecke et al., 2008; Boyko and Kovalchuk, 2010).

Although highly variable and apparently very vulnerable to experimental conditions, effects on somatic ICR rates in the progeny of UV-irradiated or pathogen infected Arabidopsis parents have been reported, indicating some kind of parental effect that is passed via the gametes to the next generation (Kovalchuk et al., 2003; Molinier et al., 2006, Pecinka et al., 2009). It was observed that this presumably stress-induced response was largely transmitted through the female parent (Boyko and Kovalchuk, 2010). We also saw that somatic mutation rates in the progeny increase with the age of the female parent (Figs. 3, 5, 6, 7), which may indicate the involvement of similar processes. Alternatively, the pronounced maternal effects we observed may be related to the maternal inheritance of mitochondria or plastids. Indeed, it has been proposed that mitochondrial impairments, which accumulate with age, are an important factor for aging in animals (reviewed in Bereiter-Hahn, 2013).

An increase in the microsatellite instability rates is known to go up with increased plant age and it was found that the DNA polymerase activity decreases with increasing plant age (Golubov et al., 2010). Impaired activity of DNA polymerase fidelity, DNA polymerase proof-reading and MMR are potential sources of replication errors. Frameshift mutations can arise as a result of replication errors involving strand slippage (Martina et al., 2012). Our results show an increase of frameshift mutation events in the progeny with increasing parental age, with a stronger effect of female parental age (Fig. 3). These changes might be caused by age-dependent maternal effects on DNA polymerase fidelity, proof-reading activity, or MMR efficiency in the progeny.

Although not significant we observed a trend towards more C→T transitions with increasing parental reproductive age that was not observed for T→G transversions (Fig. 4). An possible increase of C→T base substitution events may be related to the cytosine methylation as methyl-cytosine is prone to deamination (Mugal and Ellegren, 2011). It is possible that this increase is due to higher methyl-cytosine

deamination rates in the progeny of old parents or that their genome is more heavily methylated than that of progeny from young parents.

Double strand break repair involves homologous recombination and non-homologous end joining (NHEJ). Homologous recombination repairs DSBs in the G2/M phase of cell cycle because it requires a sister chromatid, while NHEJ is predominant in the G1/S phase (Mao et al., 2012). Our results show a significant decrease of somatic ICR events in the progeny with increasing parental age (Fig. 5B). Previous studies have shown that somatic ICR decreases with Arabidopsis plant age while NHEJ rates increase (Boyko et al., 2006), concomitant with elevated expression levels of KU70, a protein involved in NHEJ (Golubov et al., 2010). It is thus possible that the state causing the decrease of ICR in old plants is transmitted to the next generation, which could also explain the increase in DSBs in the progeny of older parents (Fig. 7). It was shown that ICR rates increase with the length of the recombination intron substrate present in the *uidA* gene (Li et al., 2004). ICR rates are lower in transgenic line R2L1 carrying a 418 bp intron as compared to line R3L30 having 589 bp of intron. This lower ICR rate might explain why we did not observe significant decrease in R2L1. Alternatively, the ICR reporter construct may be inserted in a region of the genome that is less affected by parental age.

Transpositions are known to increase exponentially with an animal's age (Nikitin and Shmookler Reis, 1997). In *Caenorhabditis elegans*, an age-dependent increase of transposition rates has been observed, and in *Drosophila melanogaster* an increase of transposition rates is associated with a decrease in life span (Egilmez and Shmookler Reis, 1994). Our results show a significant rise of transposition events in the progeny as the parental age increases, and this effect was more prominent with old female parents (Fig. 6). Thus, the effects on transposition are stronger via the female parent. It is possible that parentally inherited small RNAs are involved in controlling transposition events in the progeny (Mosher et al., 2010; Autran et al., 2011; Olmedo-Montfil et al., 2011; Calarco et al., 2012; Ibarra et al., 2012) but such parental effects have not yet been demonstrated at the molecular level.

While the application of high-throughput whole-genome sequencing technology has allowed the identification of *de novo* germline mutations in an unbiased manner (e.g. Ossowski et al., 2010; Campbell et al., 2012; Lee et al., 2012; Ness et al., 2012), this technology would not allow the detection of the extremely rare somatic mutation events occurring in a population of offspring, which we could analyze using tailor-made mutation detector lines. Whole exome sequencing has been used to identify rare somatic mutations in endometrial tumors in humans (Le Gallo et al., 2012), which was only possible because they concentrated on limited portions of clonally propagated tumorous cells. Thus, our study presents a unique view on how parental reproductive age affects mutation rates in the progeny, which revealed clear parental effects, often in a parent-of-origin-dependent manner.

Interestingly, the effect of the maternal parent's age is usually more pronounced, which may be related to the manifold ways the mother can potentially influence the next generation during seed development through the deposition of bioactive molecules in the gametes, hormonal signals, and nutrient provisioning. With increasing age, small organic molecules, RNAs and/or proteins may not be deposited in the appropriate amounts in the gametes, having an impact on the somatic mutation rate in the progeny. The unequal deposition of bioactive molecules in sperm and egg cells may account for the parent-of-origin-dependent effects we observed. Alternatively, the hereditary material itself may be affected by age and this altered chromatin state could be transmitted to the progeny, thereby altering mutation rates directly and/or by affecting the expression of genes involved in creating or repairing mutations. Finally, it is also possible that resource allocation to ovules decreases in older mothers thus exerting an effect on seed development and possibly mutation rates in the progeny after germination. Indeed, leaf age has an impact on the nutrient translocation capacity as the CO_2 fixed per unit of organic matter and the efficiency of transport in older leaves is considerably lower than in younger ones (Shiroya et al., 1961; Silvius et al., 1978).

CONCLUSION

Little is known about the effect of parental reproductive age on somatic mutation rates in the progeny of any organism. In this study we found that (1) Frameshift mutations and transposition rates increase with parental age, with a stronger effect through the maternal line (2) Although base substitution rates are not significantly affected by parental age, in general C→T transitions are higher than T→G transversions. (3) The rate of ICR events may depend on the size of inverted intron in the transgene. If the intron size is small, the ICR rate does not change but if the intron is large ICR rates decreases with parental age, but increases when the female parent is older. The overall decrease of ICR events in the progeny of older parents may result in the higher occurrence of DSBs we observed. (4) Uniformly, the female's reproductive age has a stronger effect than the male's, implying that mutation rates in the progeny depend on a parent-of-origin effect.

MATERIAL AND METHODS

Plant Material

Base substitution detector lines $166_{G\rightarrow T}$ and $1390_{T\rightarrow C}$, were a gift from Igor Kovalchuk (University of Lethbridge, Canada) and Anna Depicker (Ghent University, Belgium), respectively

(Kovalchuk et al., 2000; Van der Auwera et al., 2008). Transgenic ICR lines (R2L1 and R3L30), carrying inverted catalase introns in *uidA* gene, and frame shift detector line (G10) were a gift from Francois Belzile (University of Laval, Canada) (Li et al., 2004; Azaiez et al., 2006). The transposition detection line harboring the transposable *Tag1* element was a gift from Nigel M. Crawford (UC San Diego, USA) (Liu et al., 1998).

Plant Growth Conditions

Seeds were surface sterilized with 70% ethanol, followed by 0.5 % bleach treatment for 3 min. To remove traces of bleach, seeds were washed thrice with sterile water and plated on autoclaved Murashige and Skoog media (MS, with 3% sucrose), pH 5.7, containing 0.05% Plant Preservative Mixture (Biogenuix Medsystem Pvt. Ltd., New Delhi, India). Seed germination was synchronized by cold treatment of MS plates at 4°C for 48 h in dark. MS plates were moved to plant growth chambers, having a uniform light intensity of 8000 lx (under a 16-h light/8-h dark cycle). The temperature of the growth chamber (Percival CU-36L6) was maintained at 22°C throughout the course of experimentation, and the humidity inside the plant growth chambers was set to 80%. Two to three week old seedlings were transferred from MS plates to soil cups inside the growth chamber (Percival AR-36L3) to carry out manual cross- and manually self-pollination experiments. The soil mixture consisted of equal proportions of garden soil, peat, perlite, and vermiculite (Keltech Energies Ltd., Bangalore, India).

Self- and Cross-pollination

For cross-pollinations, flower buds prior to pollen maturation were emasculated. 48 h after emasculation, the stigmatic surface of each bud was checked for accidental deposition of pollen grains and such buds were discarded; only pollen-free stigmas were used for pollinations. Pollen from flowers of the same age was used in self-pollinations, while pollen was put on the stigma of plants of a different age in cross-pollinations. Differently colored threads were used to mark emasculated and crossed buds of different age groups. Between 10 and 25 crosses were performed in three independent replicates. Crossing experiments were performed at 22°C and seed material was harvested 16 days after pollination.

Histochemical Staining for GUS Activity

Blue spots visualized by an assay for GUS activity (Fig. S1), reflecting base substitution reversions, ICR events, frameshift mutations, and transposition events, were counted under a light microscope (Leica KL300). Histochemical staining was performed with 2-3 week-old Arabidopsis seedlings as described by Jefferson and colleagues (1987).

Ploidy Analysis by Flow Cytometry

Ploidy analysis was carried out following the protocol of Dolezel et al. (2007). Four to seven biological replicates were used to determine the percentage of nuclei with 2X, 4X, and 8X ploidy and the average number of genomes per nucleus in the progeny of different parental age. 60 mg leaf tissue of a three-week old seedling were chopped with a razor blade in a petri dish containing 1 mL of ice-cold Otto solution I (0.1 M citric acid and 0.5% [v/v] Tween 20; Sigma-Aldrich) kept at 4°C. Tomato (*Solanum lycopersicum* cv.Stupicke) was used as the internal control. The homogenate was mixed by pipetting, and filtered through a 42-µm nylon mesh. The filtrate was centrifuged at 200 *g* for 5 min to sediment the nuclei, and the pellet was resuspended in 40 mL of Otto I solution. We added 160 mL Otto II solution (0.4 M $Na_2HPO_4.12H_2O$), treated the samples with 50 µg/ml of RNase and stained the nuclei with 50µg/ml of propidium iodide. After staining, the samples were analysed using a BD FACSVerse flow cytometer (Becton, Dickinson and Company, USA). Data analysis was carried out by FCS Express 4 Plus De Novo Software (Glendale, CA, USA) and BD FACSuite software (Becton, Dickinson and Company, USA).

Cell Size and Cell number Analysis by Scanning Electron Microscopy

For scanning electron microscopy of leaves, a wax impression of plant tissue was prepared according to the protocol of Beermann and Hulskamp, 2010. We dissected the fourth true leaf (excluding the cotyledons) of a 3-week old Arabidopsis seedling derived from parents of different ages and deposited onto them a waxy dental material to generate an impression (Coltene PRESIDENT light body, Coltene AG, Altstaetten, Switzerland). After 5 min, when the wax had hardened, the leaves were gently removed. The negative mould of the leaf was filled with Spurr resin and left overnight for polymerization. The resin containing the leaf impression was removed carefully and coated with gold using a sputter coater (OC Oerlikon, Balzers, Liechtenstein). Resins were mounted on scanning electron microscope (SEM) stubs with double-sided sticky tape and the images were taken with FEI Quanta 200 SEM (Hillsboro, Oregon, USA) under 20 kV voltage and 70 Pa pressure. The total adaxial leaf surface area was analyzed using the SEM images captured at 50X magnification. To estimate cell size and cell number, images were taken at different positions of the leaf at 500X magnification, which corresponds to an area of 258929 μm^2. The average cell size was estimated by dividing the number of cells observed in an area of 500X magnification at different positions of the leaf. To calculate the total number of adaxial epidermal cells, the total area of the leaf was divided by fixed area of 500X magnification and multiplied by number of cells present in an area of 500X magnification. The number of cells and the total area of the leaf were

414 analyzed by ImageJ. 4 to 16 biological replicates were taken to determine the average number of adaxial
415 epidermal cell on the 4th true leaf.
416
417 **Estimating Correction Factors to Calculate Mutation Rates**
418 To calculate mutation rates, the average number of GUS spots per plant was divided by copy
419 number of the transgene (Kovalchuk et al., 2000). Because the number of cells and average ploidy per
420 nucleus are not identical, the total genome number will not be the same among different progenies. Hence
421 mutation rates were corrected by considering the change in number of adaxial epidermal cells of the
422 fourth leaf. The ploidy per nucleus in three-week old seedlings (derived from parents 43, 48, and 53
423 DAS) were compared with seedlings derived from self-crossed individuals at 38 DAS. To study maternal
424 age effects, progenies of young mothers (38x48 and 43x53) were compared to progenies derived from
425 older mothers (48x38 and 53x43).
426 The correction factor was calculated as
427 Titer = $(P_H*C_H)/(P_Y*C_Y)$
428 Where P_Y = is the average ploidy per nucleus. Here progenies derived from young parents (38
429 DAS) were used to calculate the differences in parental age. To assess the role of reciprocal crosses,
430 seedlings from 38x48 and 43x53 DAS parents were used.
431 P_H = is the average ploidy per nucleus in progenies derived from aged parents (43, 48 and 53 DAS
432 for selfing) and older maternal age (48x38 and 53x43).
433 C_Y = is the average number of adaxial epidermal cell in the fourth leaf of progenies derived from
434 young parents.
435 C_H = is the average number of adaxial epidermal cells in the fourth leaf of progenies derived from
436 older parents.
437 Mutation rate = GUS/titer
438 Where GUS = average number of GUS spots per plant.
439 In few instances, parents were one day older than controls. For instance, for calculating
440 transposition rates, the parental ages were 44 and 54 DAS; however, the correction factor was based on
441 seedlings derived from parents 43 and 53 DAS, respectively. Similarly, the parental age for frameshift
442 mutation (G10), intra-chromosomal recombination (R2L1), and base substitution lines was 49 DAS but
443 the correction factor was based on seedlings derived from parents 48 DAS. A difference of one day is
444 much smaller than the age difference we compared, such that we expect only a negligible influence on the
445 estimation of mutation rates.
446

Comet Assay to Study Double Strand Breaks at Different Parental Age

With little modifications, a neutral comet assay was performed as per the protocol recommended by the manufacturer (Oxiselect Comet Assay Kit, Cell Biolabs, Inc. San Diego, USA). 50-100 mg tissue of three week old Arabidopsis seedlings from different parental ages (38, 48, 53, 43x53, and 53x43 DAS) were chopped with a razor blade in 1ml Otto I solution. Subsequently, the sample was centrifuged at 300 rpm for 5 min and the pellet was dissolved in 1X PBS containing 20 mM EDTA. The dissolved sample was mixed with warm low-melting agarose in a ratio of 2:5, and poured onto a slide coated with agarose. A cover slip was placed on top of the sample mix and the slide was stored at 4˚C in dark for 15 min horizontally. Then slides were transferred to chilled lysis buffer for 30-60 min at 4˚C in dark. The slides were subsequently transferred to cold alkaline solution for 30 min at 4˚C in dark. The samples on the slide were treated with chilled TBE buffer for 5 min and transferred to a horizontal electrophoresis chamber containing chilled TBE buffer. Electrophoresis was carried out at 1Volt/cm for 15 min. Thereafter, the slides were washed thrice with de-ionized water and treated with 70% ethanol for 5 min. After air drying, the sample was stained with vista green and comets were observed using an upright fluorescent microscope (Nikon Eclipse 80i) fitted with a FITC filter. Comet analysis was carried using the comet assay IV online software.

Statistical Analysis

Between 150 and 500, 80 and 800, 60 and 200, and 150 and 400 plants of a population were analyzed to detect base substitutions, ICR events, frameshift mutations, and transpositions, respectively. Staining was performed in at least three replicates. The total number of plants analyzed per population is indicated in the figures.

The number of GUS-spots are count data, which is why we chose a Quasi-Poisson generalized linear model (GLM) with the log link function (Nelder and Wedderburn, 1972). The Quasi-Poisson GLM was used to account for the overdispersion of the data. The linear predictors were the different age groups and their usage as female or male parent. The log of the correction factor for cell number and ploidy per leaf cell nucleus was added in the models as fixed intercept.

In all GLMs, the data from the groups were used for several comparisons. For example, in frameshift mutation line G10, 38 DAS was compared with 43 DAS, 48 DAS, 53 DAS and 43 DAS compared with 48 DAS, 53 DAS and 48 DAS compared with 53 DAS. Thus correction for multiple testing was done to maintain the family-wise error rate at 5% (Gabriel, 1969). Therefore, we adjusted *P* values with a single-step method that considers the joint multivariate *t* distribution of the individual test statistic (Bretz et al, 2010).

The results are reported with the two-sided *P* value adjusted for multiple comparisons. All statistical analyses were carried out in R (R Developmental Core Team, 2010). To adjust the *P* values for multiple testing, the R package multcomp was used with the test specification "single-step" (Bretz et al., 2010). Graphs were produced using the ggplot2 package (Wickham, 2009).

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Figure Legends

Figure 1. Cell size and cell number. (A, B, E) Cell size does not change with parental age. (C, D, F) Cell number decreases with parental and female reproductive age, resulting in a smaller leaf surface area. **(**A-D) SEM images of the adaxial epidermal cells of the fourth true leaf of a 3-week-old Arabidopsis seedling. (E, F) Box plots of cell size and the average number of adaxial epidermal cells from seedlings derived from parents of different ages. The numbers at the bottom of the graph show the biological replicates analyzed. * – $P< 0.05$; ** – $P< 0.01$; *** – $P< 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

Figure 2. Differences in the nuclear ploidy among the progenies from different age groups. (A) Percentage of diploid, tetraploid, and octoploid nuclei in leaves; different shades of grey indicate different ploidy. (B) Average ploidy per leaf cell nucleus. For each cross, a box plot of the average ploidy per leaf cell nucleus is drawn. The numbers at the bottom of the graph show the biological replicates analyzed. * – $P< 0.05$; ** – $P< 0.01$; *** – $P< 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

Figure 3. Frameshift mutation rates in Arabidopsis derived from line G10. Frameshift mutation frequency in the F1 progeny of selfed and reciprocally crossed parents from different age points. Point predictions and 95% confidence intervals for frameshift mutation rates are drawn. Numbers at the bottom of the graph indicate the number of seedlings analyzed. * – $P< 0.05$; ** – $P< 0.01$; *** – $P< 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

Figure 4. Base substitution rates in F1 progeny of selfed and reciprocally crossed parents from different ages. (A) Line$166_{G\rightarrow T}$. (B) Line $1390_{T\rightarrow C}$. Point predictions and 95% confidence intervals for base substitution rates are drawn. Numbers at the bottom of the graph indicate the number of seedlings analyzed. * – $P< 0.05$; ** – $P< 0.01$; *** – $P< 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

Figure 5. Intra-chromosomal recombination (ICR) frequencies. (A) ICR frequency for line R2L1 in the F1 progeny of selfed and reciprocally crossed parents from different age points. (B) ICR frequency for line R3L30 in the F1 progeny of selfed and reciprocally crossed parents at different age points. Point predictions and 95% confidence intervals for ICR rates are drawn. Numbers at the bottom of the graph indicate the number of seedlings analyzed. * – $P< 0.05$; ** – $P< 0.01$; *** – $P< 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

Figure 6. Transposition rates in Arabidopsis plants derived from the *Tag1* line. Transposition frequency in the F1 progeny of selfed and reciprocally crossed parents from different age points. Point predictions and 95% confidence intervals for transposition rates are drawn. Numbers at the bottom of the graph indicate the number of seedlings analyzed. * – $P< 0.05$; ** – $P< 0.01$; *** – $P< 0.001$; no asterisk – no

706 significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are
707 given in "Statistical Analysis".
708
709 **Figure 7.** Double strand DNA breaks in seedlings derive from parents of different age. Percentage of tail
710 DNA was quantified using the neutral comet assay in the F1 progeny of self- and reciprocally crossed
711 parents from different age groups. Point predictions and 95% confidence intervals for the percentage of
712 tail DNA are drawn. Numbers at the bottom of the graph indicate the number of comets analyzed. * – $p <$
713 0.05; ** – $p < 0.01$; *** – $p < 0.001$; no asterisk – no significant difference. *P* values are corrected for
714 multiple testing. More details concerning the analysis are given in "Statistical Analysis".
715
716 **Table Legend**
717 **Table 1.** Normalization of mutation rates by factoring differences in cell number and ploidy per nucleus.
718 Progenies of different parents were analyzed for their adaxial epidermal cell count and their ploidy per
719 leaf cell nucleus to obtain a correction factor. The coefficient of interquartile range (CIQR) was
720 calculated as a nonparametric measurement of variance in the style of the coefficient of variance (CV).
721 The relative number of cells and the relative ploidy are the normalization values for older parental age
722 (43, 48 and 53 days after sowing (DAS)) compared with youngest parental age (38 DAS), and for
723 reciprocal crosses, the female with higher age (48 x 38 and 53 x 43) was compared with younger age (38
724 x 48 and 43 x 53). The correction factor was calculated by multiplying the two normalization values. This
725 correction factor was used to correct the number of GUS spots before analysis. n – Number of plants
726 analyzed; Median – median of measurements; CIQR – interquartile range/median.
727
728
729
730 **Supplementary Figure Legends**
731 **Supplemental Figure S1.** Functional GUS reversion event resulting in blue spot on a true leaf of a three
732 week old Arabidopsis seedling.
733
734 **Supplemental Figure S2.** Total leaf surface area in the progenies derived from the parents of different
735 age groups. For each cross a box plot is drawn. The numbers at the bottom of the graph show the
736 biological replicates analyzed. * – $P< 0.05$; ** – $P< 0.01$; *** – $P< 0.001$; no asterisk – no significant
737 difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in
738 "Statistical Analysis".

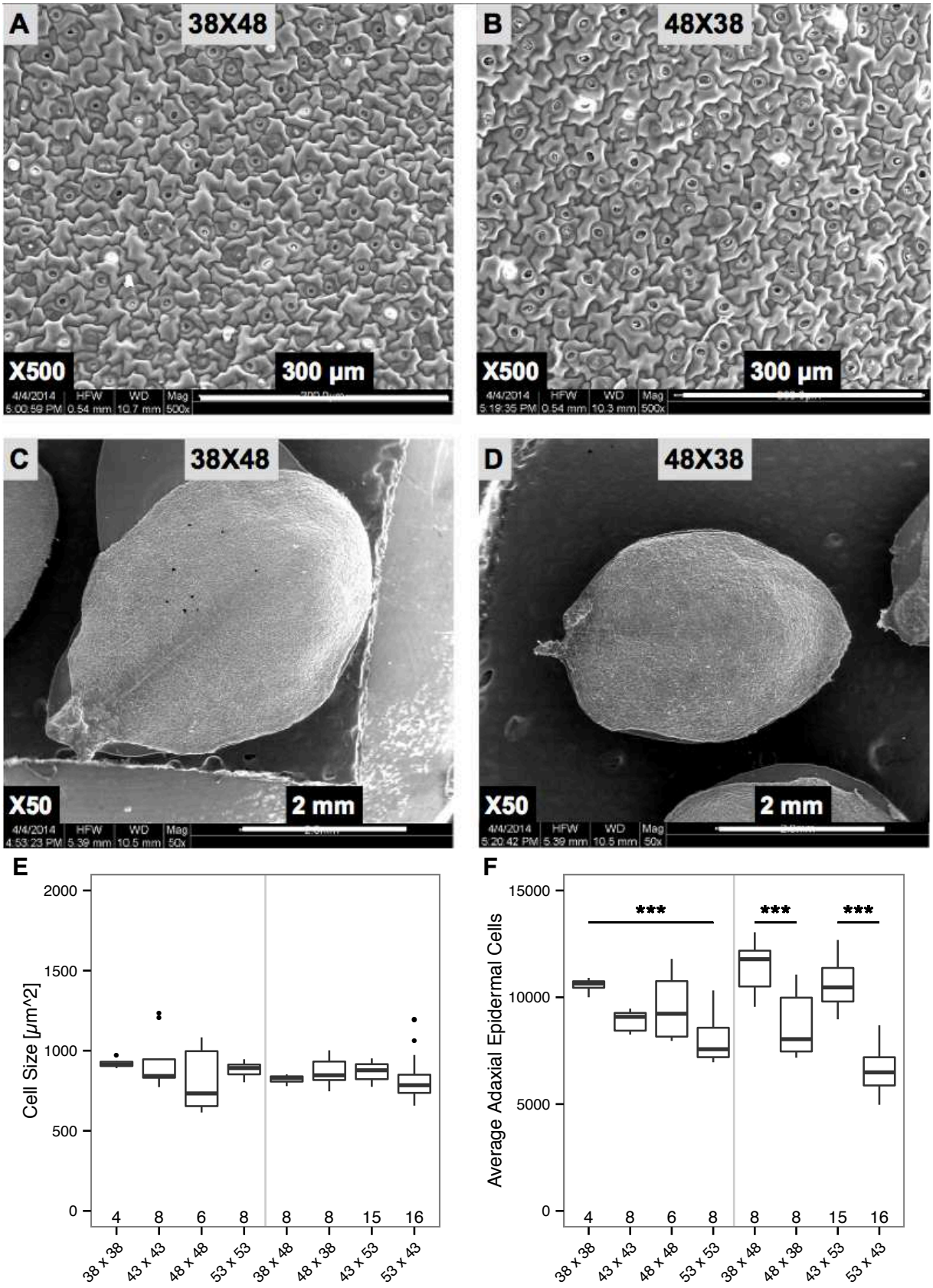


Figure 1

Figure 1. Cell size and cell number. (A, B, E) Cell size does not change with parental age. **(C, D, F)** Cell number decreases with parental and female reproductive age which results in a smaller leaf surface area.

(A-D) SEM images of the adaxial epidermal cells of the fourth true leaf of a 3-week-old Arabidopsis plant. **(E, F)** Box plots of cell size and the average number of adaxial epidermal cells from seedlings derived from parents of different ages. The numbers at the bottom of the graph show the biological replicates analyzed. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

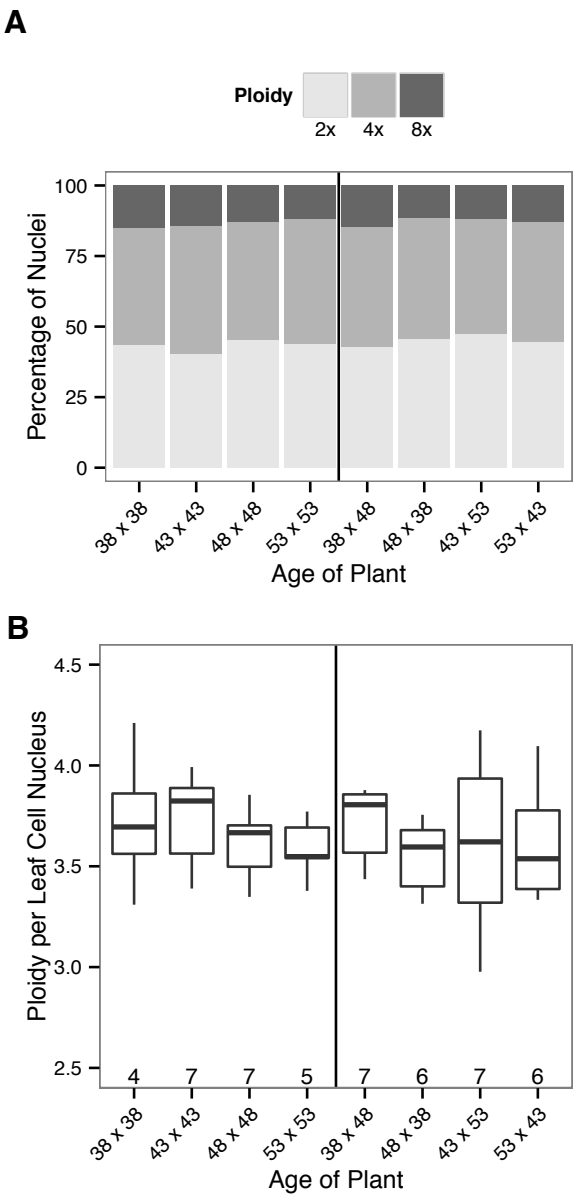


Figure 2

Figure 2. Differences in the nuclear ploidy among the progenies derived from parents of different age groups. (A) Percentage of diploid, tetraploid, and octoploid nuclei in leaves with different shades of grey indicating different ploidy. **(B)** Average ploidy per leaf cell nucleus. For each cross, a box plot of the average ploidy per leaf cell nucleus is drawn. The numbers at the bottom of the graph show the biological replicates analyzed. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

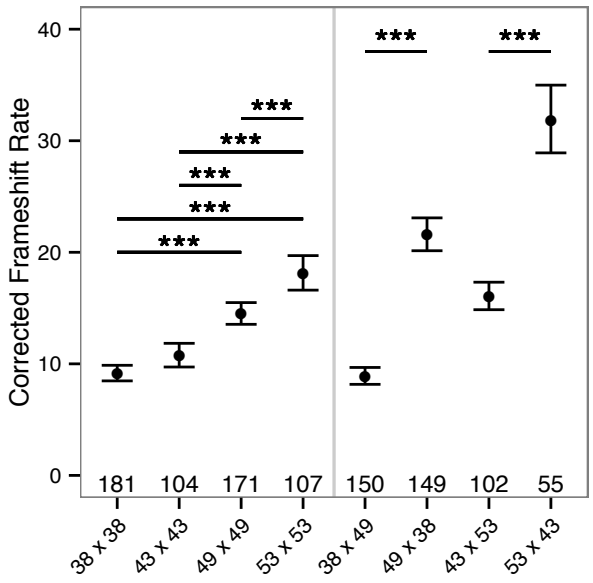


Figure 3

Figure 3. Frameshift (FS) mutation rates in Arabidopsis derived from line G10. FS mutation frequency in the F1 progeny of selfed and reciprocally crossed parents from different age points. Point predictions and 95% confidence intervals for FS mutation rates are drawn. Numbers at the bottom of the graph indicate the number of seedlings analyzed. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

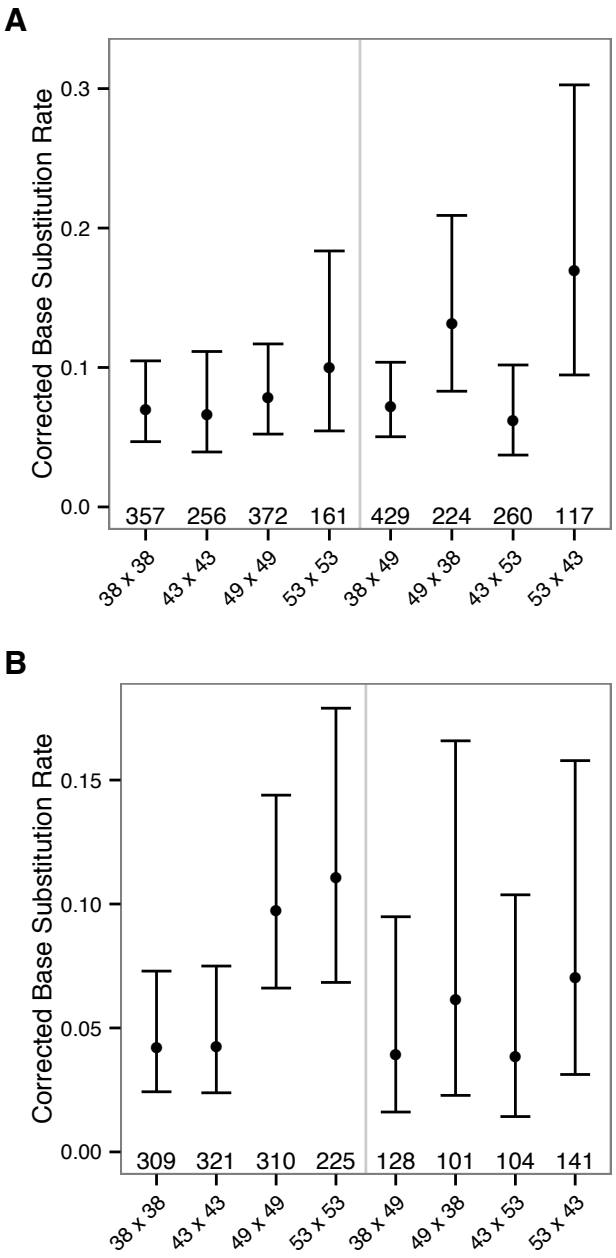


Figure 4

Figure 4. Base substitution rates in F1 progeny of selfed and reciprocally crossed parents from different ages. (A) Line$166_{G\rightarrow T}$. **(B)** Line $1390_{T\rightarrow C}$. Point predictions and 95% confidence intervals for base subsitution rates are drawn. Numbers at the bottom of the graph indicate the number of seedlings analyzed. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

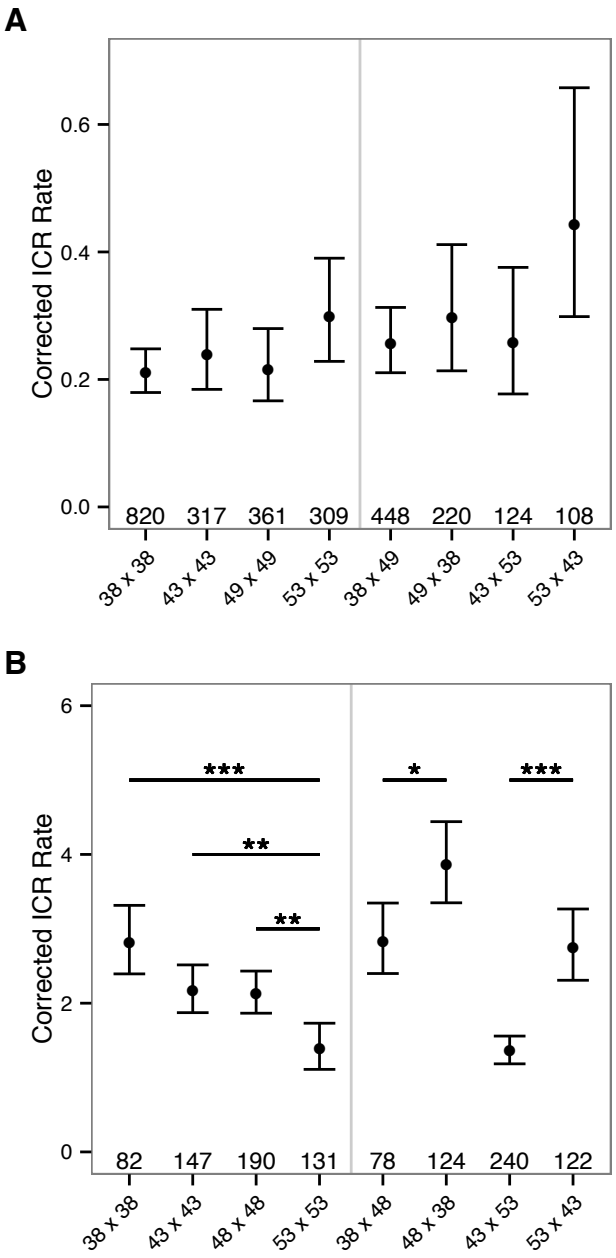


Figure 5

Figure 5. Intra-chromosomal recombination (IRC) rates. (A) ICR rates for line R2L1 in the F1 progeny of selfed and reciprocally crossed parents from different age points. **(B)** ICR rates for line R3L30 in the F1 progeny of selfed and reciprocally crossed parents at different age points.

Point predictions and 95% confidence intervals for ICR rates are drawn. Numbers at the bottom of the graph indicate the number of seedlings analyzed. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

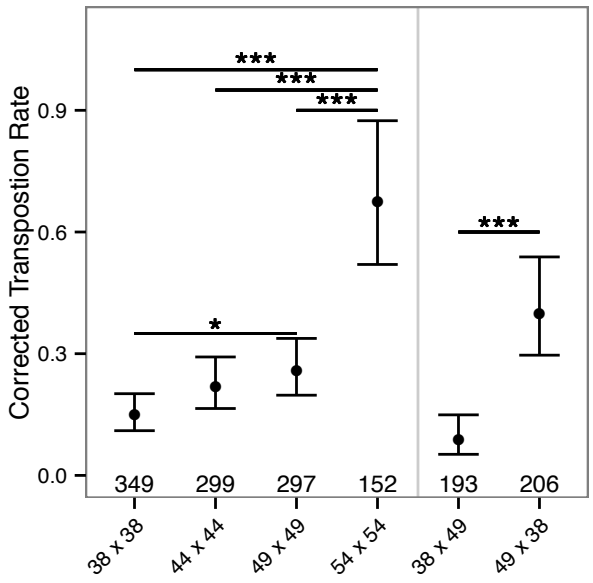


Figure 6

Figure 6. Transposition rates in Arabidopsis plants derived from the *Tag1* line. Transposition frequency in the F1 progeny of selfed and reciprocally crossed parents from different age points. Point predictions and 95% confidence intervals for transposition rates are drawn. Numbers at the bottom of the graph indicate the number of seedlings analyzed. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

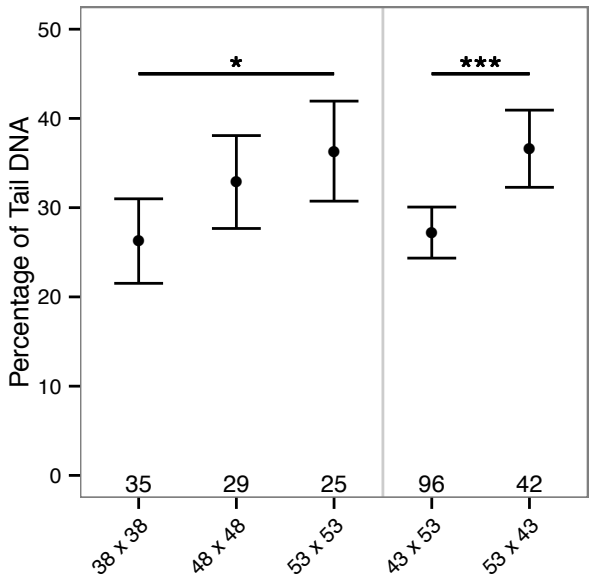
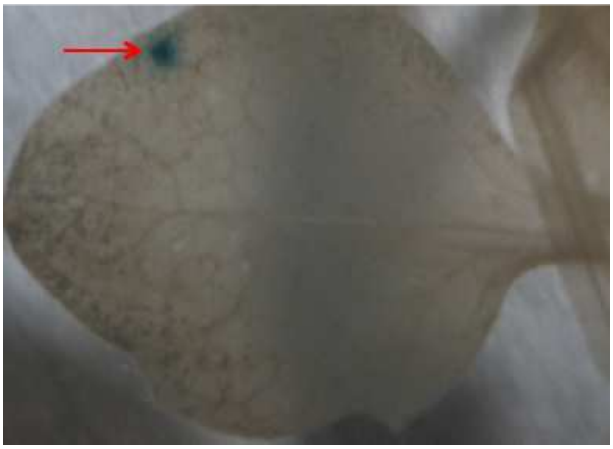
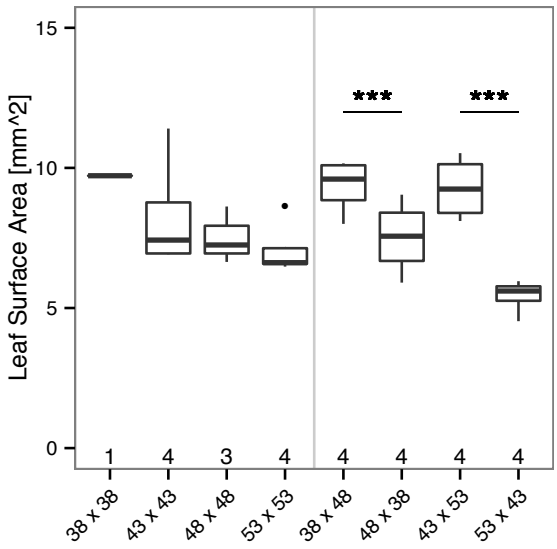


Figure 7

Figure 7. Double strand DNA breaks in seedlings derived from parents of different age. Percentage of tail DNA was quantified using the neutral comet assay in the F1 progeny of self- and reciprocally crossed parents from different age groups. Point predictions and 95% confidence intervals for the percentage of tail DNA are drawn. Numbers at the bottom of the graph indicate the number of comets analyzed. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis"



Supplementary Figure S1. Functional GUS reversion event resulting in blue spot on a true leaf of a three week old Arabidopsis seedling.



Supplementary Figure S2. Total leaf surface area in the progenies derived from the parents of different age groups. For each cross a box plot is drawn. The numbers at the bottom of the graph show the biological replicates analyzed. * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; no asterisk – no significant difference. *P* values are corrected for multiple testing. More details concerning the analysis are given in "Statistical Analysis".

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